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PRINCIPAL INVESTIGATOR: Kathryn Schwertfeger, B.S.
Dr. Steven Anderson

CONTRACTING ORGANIZATION: University of Colorado
Health Sciences Center
Denver, Colorado 80262

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6. AUTHOR(S)
Kathryn Schwertfeger, B.S.
Dr. Steven Anderson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

E-MAIL:
Kaylee.stack@uchsc.edu

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13. ABSTRACT *(Maximum 200 Words)*

Breast cancer can be a result of both uncontrolled cellular proliferation and a failure of cells to undergo apoptosis, a genetically controlled process by which cells regulate their own survival. Apoptosis in the mammary gland has been well documented during both virgin development and involution. Numerous studies have shown that the serine/threonine protein kinase Akt can suppress apoptosis. Akt is activated by numerous growth factors, including prolactin, which are involved in mammary development and function. One goal of this proposal is to determine the signaling pathways involved in prolactin-induced Akt activation. Results indicate that prolactin-induced Akt activation is mediated through both the PI3-kinase and src-like kinase pathways. In addition, the downstream effects of Akt activation will also be examined. Adenoviruses expressing different Akt constructs have been made and will be used to address this issue. The main goal of this proposal is to determine the role of Akt in the development of mammary cancer. Transgenic mice expressing a constitutively active form of Akt were produced that show a delay in involution caused by suppression of apoptosis of the secretory epithelial cells. We predict that this suppression of apoptosis will result in the development of lesions that may lead to tumors.

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Kathryn Schwartz 7/27/00

PI - Signature Date

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Introduction

Cancer is thought to result from both uncontrolled cell proliferation as well as the failure of specific cells to undergo apoptosis. The serine/threonine protein kinase Akt has been shown by numerous studies to suppress apoptosis (1-4). Therefore, we have hypothesized that overexpression of Akt may lead to the inability of cells to undergo programmed cell death when necessary, thus resulting in hyperplasias and tumors. Akt is activated by growth factors that are involved in development of the mammary gland, such as insulin-like growth factor-I (2). We have also shown that Akt is activated in response to prolactin, which is involved in mammary gland development and function. The first two tasks proposed in the grant involve the characterization of the pathway that mediates prolactin-induced activation of Akt. The third task focuses on the potential role of Akt during mammary gland development and tumorigenesis. As described in this summary, transgenic mice have been produced that express a constitutively active mutant of Akt in the mammary gland. Analysis of these mice indicate that the activated Akt can suppress apoptosis during mammary gland involution, which may eventually result in hyperplasias and/or tumors.

Body

The focus of the research supported by this grant is on the protein kinase Akt and its potential role in tumorigenesis in the mammary gland. Progress has been made in all three tasks proposed in the Statement of Work. Task 1 focuses on characterizing the signaling molecules that are involved in mediating Akt activation in response to prolactin. This was achieved by treating cells with chemical inhibitors of specific molecules and examining the ability of prolactin to induce activation of Akt. Nb2 cells, a prolactin-dependent cell line, were treated with wortmannin, which inhibits activation of PI3-kinase, followed by stimulation with prolactin. Lysates were analyzed by immunoblot analysis using an antibody that recognizes the phosphorylated, activated form of Akt. Cells treated with wortmannin and prolactin show a decrease in the amount of Akt phosphorylation compared to cells treated with prolactin alone or ethanol and prolactin (Figure 1). This experiment was also performed using the human breast cancer cell line, T47D, with similar results (data not shown). These experiments indicate that Akt activation in response to prolactin is mediated through the PI3-kinase pathway.

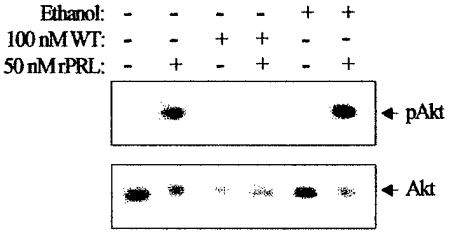

Figure 1. Akt activation is inhibited following wortmannin treatment of Nb2 cells. Nb2 cells were cultured overnight in the absence of prolactin, and then stimulated with 50 nM rPRL and either 100 nM wortmannin or ethanol for 30 minutes. The cells were lysed and 50 μg total protein were analyzed on a polyacrylamide gel. The proteins were then transferred to membrane and immunoblotted with an antibody to the phosphorylated form of Akt (upper panel). The blot was re-probed with an antibody to total Akt to indicate equal loading (lower panel).

Experiments have shown that prolactin induced activation of PI3-kinase is mediated by members of the src-like kinase family (5). Therefore, the effect of the inhibitor PP1, which inhibits the activity of the src-like kinase family, was also examined. Nb2 cells treated with PP1 and prolactin show a decrease in Akt activation compared to cells treated with prolactin alone or prolactin and DMSO (Figure 2). This indicates that activation of Akt by prolactin is also mediated through the src-like kinases. Further studies are required to demonstrate that the observed effects are specific and not the consequence of non-specific activity of the inhibitors on unrelated molecules. These experiments can be performed utilizing dominant negative mutants of PI3-kinase and Fyn, a member of the src-like kinase family. If the results from the inhibitor studies are specific, then expression of these constructs in the cells should result in a decrease in Akt phosphorylation.

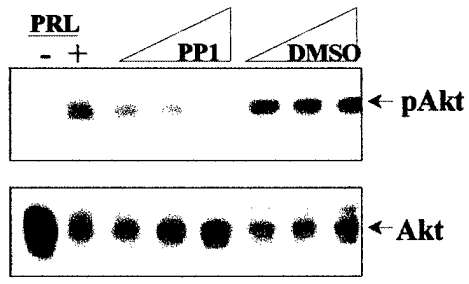

Figure 2. Inhibition of Akt activation following PP1 treatment of Nb2 cells. Nb2 cells were incubated in the presence of either PP1 (0-100μM) or DMSO for 4 hours. The cells were then stimulated with 50 nM rPRL for 30 minutes and lysed using NP40 lysis buffer. 50 μg total protein were analyzed on an 8% SDS-PAGE gel. The proteins were transferred to membrane and immunoblotted with an antibody that recognizes the phosphorylated form of Akt (upper panel). The blot was re-probed with an antibody to total Akt to indicate equal loading (lower panel).

The aim of Task 2 is to characterize the effects of a constitutively active mutant of Akt in cell culture. This construct was made by fusing the myristoylation sequence of Src onto the amino terminus of Akt (6). This localizes Akt to the membrane where it can be constitutively phosphorylated and thus activated. Due to difficulties with stable transfection of the Akt constructs into cells, three Akt constructs, wild-type (WT), kinase inactive (K-) and constitutively active (Myr), were subcloned into adenoviral vectors. The resulting adenoviral constructs were used to infect 293 cells to make adenoviruses expressing these Akt constructs. The adenoviruses were transduced into the mouse mammary epithelial cell line, HC-11. Lysates were prepared from these cells, analyzed on a polyacrylamide gel, and immunoblotted with an antibody to the HA epitope tag. As indicated in Figure 3A, all three adenoviruses were able to transduce HC-11 cells and express the Akt constructs. To determine whether the myr-Akt construct was constitutively activated, HC-11 cells were transduced with the adenoviruses, Akt was immunoprecipitated, and the ability of Akt to phosphorylate a known substrate, glycogen synthase kinase –3 (GSK-3), was measured. As shown in Figure 3B, even in the absence of growth factors, the myr-Akt and WT-Akt transduced cells were able to phosphorylate GSK-3, whereas the K-Akt and green fluorescent protein (GFP) transduced cells were not. This experiment suggests that the myr-Akt construct is constitutively activated, which is crucial for the transgenic mouse experiments described in Task 3. Further studies will address whether the presence of the myr-Akt in mammary epithelial cells will result in suppression of apoptosis. In addition, the ability of the myr-Akt to phosphorylate known substrates is also being studied in these cells.

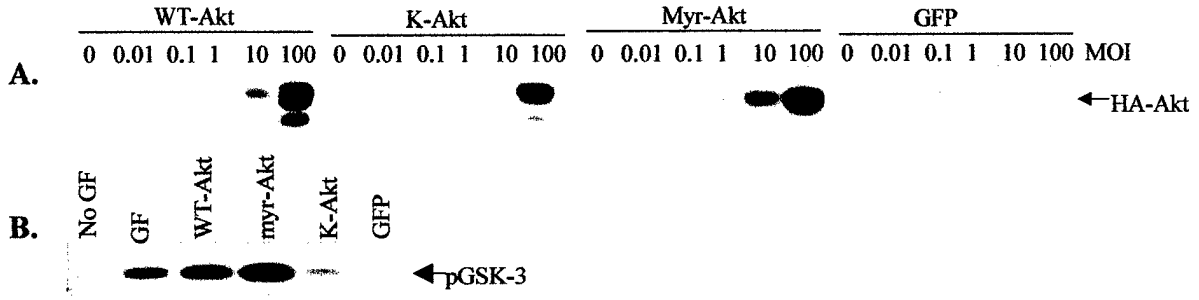

Figure 3. Analysis of Akt construct protein expression and activity in HC-11 cells following transduction of Akt adenoviruses. A) Confluent HC-11 cells were transduced in serum free media with increasing MOIs using the indicated adenoviruses followed by a 24 hour incubation in complete HC-11 media. Cells were lysed and equal amounts of protein were analyzed on an 8% polyacrylamide gel, transferred to membrane, and immunoblotted with an antibody to the HA epitope tag. B) Akt was immunoprecipitated from lysates of cells transduced with the indicated adenoviruses and starved of growth factors (GF) overnight. Immunoprecipitates were incubated with GST-GSK-3 and ATP. The complexes were analyzed on a polyacrylamide gel and immunoblotted with an antibody to phosphorylated GSK-3.

The primary focus of this project is on the role of Akt in the mammary gland. To address this question, a mouse model of mammary gland development has been used. One important question to address is whether Akt is present in the mammary gland and whether it has an expression pattern during development that suggests that it has a role during development. Mammary glands were removed from virgin, pregnant, lactating, and involuting normal mice. Protein was extracted from the tissues and analyzed by immunoblot analysis using an antibody to Akt. Figure 4 shows that Akt is present in the mammary gland at all stages analyzed, and that its expression decreases following weaning, at the beginning of involution at least through day 4. This is interesting because the peak of apoptosis during mammary gland involution is during day

4 (7). This suggests that the absence of Akt may be necessary for proper apoptosis during involution. An experiment that would address this would be to express Akt in the mammary gland during the time when it should be decreased and examine what happens during involution, which is the focus of Task 3.

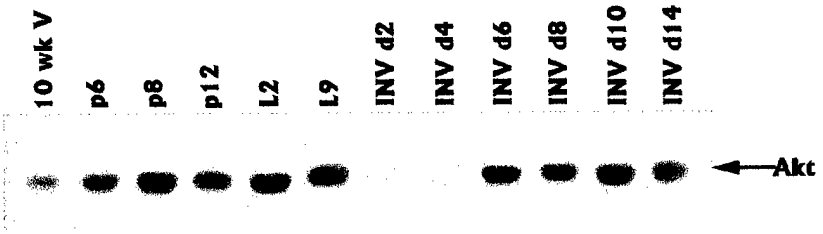

Figure 4. Akt expression during normal mammary gland development. Mammary glands were dissected from mice at the following stages: 10 week virgin (10 wk V), pregnancy at days 6, 8, and 12 (p6, p8, p12), lactation at days 2 and 9 (L2, L9) and involution at days 2, 4, 6, 8, 10, and 14 (INV d2, INV d4, INV d6, INV d8, INV d10, INV d14). The tissues were lysed and protein extracted. 50 ug whole cell lysate was analyzed on an 8% polyacrylamide gel and immunoblotted with an antibody to Akt.

The initial focus of Task 3 was to develop a transgenic mouse expressing the myr-Akt construct in mammary epithelial cells. The myr-Akt construct was subcloned into a vector containing the Mouse Mammary Tumor Virus (MMTV) promoter, which has been shown to drive transgene expression in the epithelial cells of the mammary gland (8). This construct was then injected into the pronuclei of fertilized eggs and placed into the uterus of a surrogate mother. From the 39 founder mice that were born, 9 were positive for the transgene as determined by tail DNA PCR (Figure 5A). RNA was isolated from mammary glands of mice derived from these lines on day 18 of pregnancy, and RT-PCR was performed to determine whether these mice express transgene mRNA (Figure 5B). Transgene mRNA was detected in mammary glands of mice derived from 1173, 1176, 1177, 1178, and 1699. To further explore transgene expression, protein was isolated from mammary glands at day 18 of pregnancy and immunoblotted with an antibody that recognizes the HA epitope tag. As seen in Figure 5C, mice from lines 1173 and 1176 show detectable protein expression of the transgene. These data suggest that transgene mRNA and protein are being expressed in these two lines and can be detected in the mammary glands of these mice.

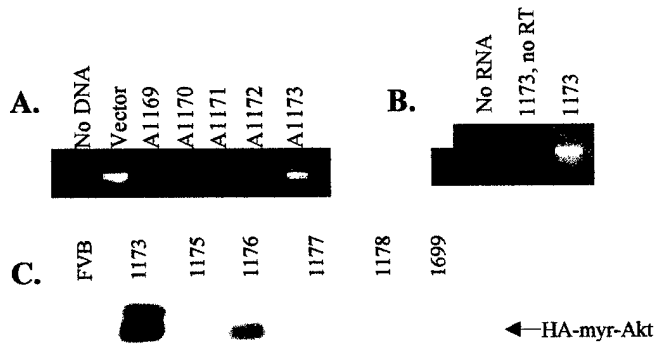

Figure 5. Development of the transgenic line expressing myr-Akt in the mammary gland. A) Tail DNA was extracted from 39 founders. PCR was performed to detect the transgene. 9/39 mice were PCR positive. B) RNA was extracted from mammary glands of mice derived from the 9 PCR positive lines. RT-PCR was performed to detect transgene RNA. C) Protein was extracted from mammary glands of PCR positive mice, analyzed on a polyacrylamide gel, and immunoblotted with an antibody to the HA epitope tag.

The expression patterns of the transgene during the stages of mammary gland development, virgin, pregnancy, lactation, and involution in line 1173 have been examined. Mammary glands were removed from mice at the described stages, protein was extracted and subjected to immunoblot analysis using the anti-HA antibody. As shown in Figure 6, transgene expression is undetectable in early virgin glands and pregnancy, but increases dramatically during lactation and remains detectable throughout involution. Although transgene expression is not detectable by immunoblotting in the virgin and pregnant stages, it might still be expressed at low levels. To address this, Northern blot analysis will be performed on these samples, which will indicate whether RNA is being expressed in virgin and pregnant mice.

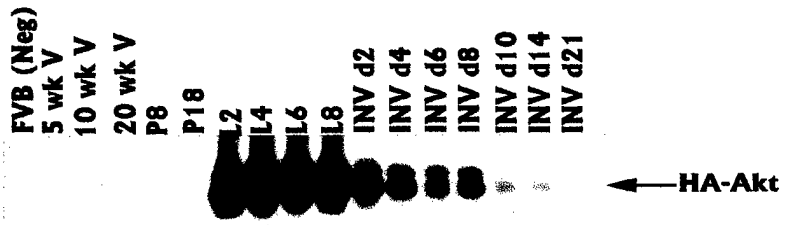

Figure 6. Transgene expression during different stages of development in line 1173. Mammary glands were dissected from mice at the indicated stages and the tissue lysed. 50 ug whole cell lysate was analyzed on an 8% polyacrylamide gel and immunoblotted with an antibody to the HA epitope tag.

One of the primary timepoints during which apoptosis occurs in the mammary gland is involution. Following weaning of the pups, between 50 and 85% of the secretory epithelial cells undergo apoptosis. One focus of this project is to determine whether expression of the constitutively active Akt will suppress apoptosis induced by weaning. Normal and transgenic mice from line 1173 were mated and gave birth. All litters were normalized to 8 pups each and were allowed to suckle for 9 days to establish full lactation. The pups were then weaned and the mothers allowed to involute for 2, 4, 6, 8, 10, and 14 days. The inguinal mammary glands were then removed, sectioned, and stained with hematoxylin/eosin. Figure 7 shows mammary gland sections from both normal (A-E) and transgenic (F-J) mice. At day 2 of involution in the normal gland, alveolar structures are visible with some apoptotic bodies present in the lumens (Figure 7A). At day 4, the alveolar structures in the normal gland are beginning to collapse and the adipocytes are beginning to return (Figure 7B). Numerous apoptotic bodies can be found in the lumens of the ducts and this is the stage during which the highest percentages of epithelial cells are undergoing apoptosis (7). This process continues until the alveoli have collapsed and the gland has been completely remodeled. By day 14, the structures in the gland resemble those of virgin glands (Figure 7E). The mammary glands of the transgenic mice at day 2 look similar to the normal glands (Figure 7A,F). However, at day 4, the alveoli in the transgenic glands have not yet begun to collapse and the gland looks significantly different than the normal gland (Figure 7B, G). At day 6, alveolar structures are still present, although it appears that the adipocytes are beginning to reappear and apoptotic bodies can be seen in the lumens (Figure 7C, H). By day 8, the alveoli are beginning to collapse and the adipocytes are reappearing, indicating that involution is occurring (Figure 7D, I). This continues and by day 14, the gland appears to be remodeled, similar to the normal day 14 gland (Figure 7E, J). However, some structures can still be visualized throughout the gland that have not undergone involution and there appears to be more epithelium than in the normal glands, suggesting that a smaller percentage of epithelial cells are undergoing apoptosis in the transgenic mice. This delay in involution was also confirmed in the 1176 line, indicating that the observed phenotype is due to

the presence of the transgene and not an effect of transgene insertion (data not shown). These data suggest that the presence of a constitutively active Akt in the mammary gland can delay mammary gland involution following weaning.

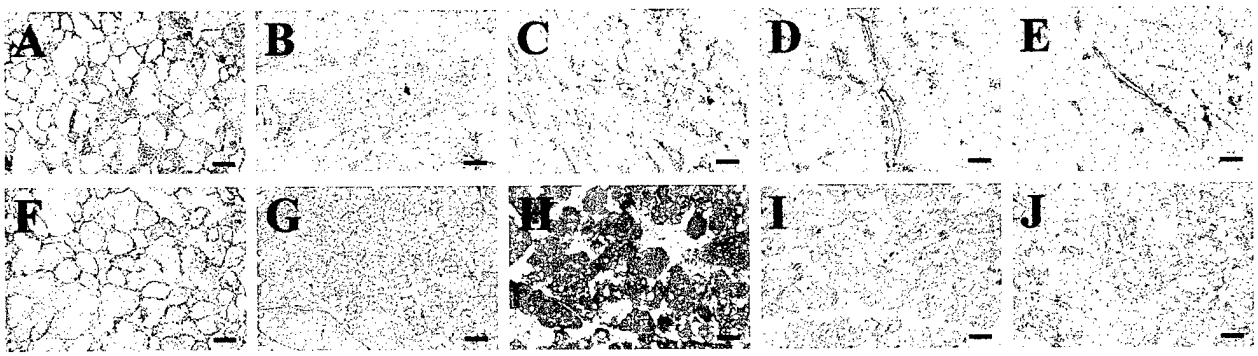

Figure 7. Involution in both normal and transgenic mice. Normal (A-E) and transgenic (F-J) female mice were mated and gave birth. Litters were normalized to 8 pups. Following 9 days of lactation, the pups were removed and the mothers were then sacrificed at day 2 (A, F), day 4 (B, G), day 6 (C, H), day 8 (D, I), or day 14 (E, J) of involution. The inguinal mammary glands were removed, fixed, sectioned, and the sections stained with hematoxylin/eosin.

Because Akt mediates cell survival, we predict that this delay in involution is due to the failure of the epithelial cells to undergo apoptosis. Therefore, we have determined the percentages of epithelial cells undergoing apoptosis in both normal and transgenic mice. First, hematoxylin/eosin stained sections were examined. The percentages of apoptotic bodies to total epithelial cells were determined in a blind study. Figure 8 shows that in the normal glands, apoptosis peaks at day 4 of involution, consistent with results seen by other investigators (7). However, the peak of apoptosis in the transgenic glands appears delayed, peaking at day 8 of involution. This suggests that myr-Akt is suppressing apoptosis of the secretory epithelial cells, resulting in delayed involution. These results are currently being confirmed using the TUNEL assay.

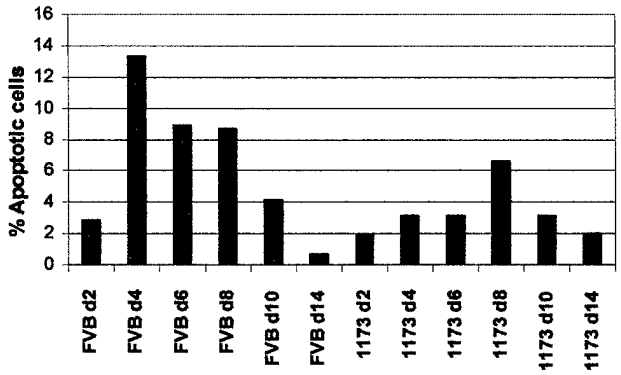

Figure 8. Suppression of apoptosis during involution in the mammary glands of transgenic mice. The number of apoptotic bodies were counted in hematoxylin/eosin stained sections of mammary glands from both normal and transgenic mice during involution. The percentage of apoptotic cells was calculated from the total numbers of epithelial cells.

Future experiments using these mice include determining whether the suppression of apoptosis can result in the development of hyperplasias and/or tumors. We predict that the incomplete suppression of apoptosis following involution may lead to the development of hyperplastic alveolar nodules, which are potentially precancerous lesions. Experiments are in progress in which the mice are being examined at the completion of involution following one or multiple pregnancies. These experiments should help to define a role for Akt in the development of mammary lesions or tumors.

Key Research Accomplishments

- Experiments using the chemical inhibitors wortmannin and PP1 indicate that prolactin-induced activation of Akt is mediated through both PI3-kinase and members of the src-like kinase family.

- Adenoviruses expressing wild-type Akt, constitutively active Akt, and kinase inactive Akt were produced and can be expressed in HC-11 cells in a dose-dependent manner.

- Akt was shown to be present in the mammary gland and decreases during the beginning of involution, indicating that the decrease in Akt expression may be important for proper apoptosis of the epithelial cells at this stage.

- The MMTV-myr-Akt transgenic mice were produced and two lines that express the transgene have been identified.

- Involution has been shown to be delayed in both lines of transgenic mice, which was shown to be a result of suppression of apoptosis of the epithelial cells.

Reportable Outcomes

Abstracts:
THE ROLE OF AKT IN PROLACTIN SIGNALING AND MAMMARY GLAND DEVELOPMENT. KS Schwertfeger, MM Richert, and SM Anderson, Program in Molecular Biology and Department of Pathology, University of Colorado, Denver, CO.
Presented as a poster at the Prolactin Gordon Conference, February, 2000.

DELAYED MAMMARY GLAND INVOLUTION IN TRANSGENIC MICE EXPRESSING ACTIVATED AKT
Steven M Anderson, Kathryn L Schwertfeger, Monica M Richert, Robert Strange. Pathology; Molecular Biology, University of Colorado Health Sciences Center; AMC Cancer Research Center, Denver, CO.
Presented as a talk by SMA at the Endocrine Society Meeting, 2000.

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THE ROLE OF AKT IN PROLACTIN SIGNALING AND MAMMARY GLAND DEVELOPMENT. KS Schwertfeger, MM Richert, and SM Anderson, Program in Molecular Biology and Department of Pathology, University of Colorado, Denver, CO.

Studies have shown that activation of the serine/threonine protein kinase Akt (also known as protein kinase B) by a number of growth factors such as insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), and interleukin-3 (IL-3) results in the suppression of apoptosis. Activation of Akt occurs in a PI3-kinase dependent manner. Because prolactin (PRL) can activate PI3-kinase, this prompted us to ask whether PRL can also mediate activation of Akt. Stimulation of two different PRL-responsive cell lines, Nb2 and T47D results in Akt activation in both a time and dose-dependent manner. Although PRL has many different roles, its predominant function is to stimulate mammary gland development and lactogenesis. Several growth factors that influence mammary gland development, EGF, IGF-I, and PRL, all actiave Akt; therefore, we decided to examine the role of Akt in mammary gland development. Using immunoblot analysis, we show that Akt is expressed in the virgin, pregnant, lactating, and involuting mammary gland. To determine the function of Akt in the mammary gland, we have developed a transgenic mouse model in which a constitutively active form of Akt is targeted to mammary epithelial cells using the mouse mammary tumor virus (MMTV) promoter. We hypothesize that the expression of the active Akt may inhibit apoptosis that normally occurs during pubertal development and involution. Founder mice were established and the expression of the transgene has been determined using both RT-PCR and immunoblot analysis. Initial studies suggest that involution does not occur normally in the transgenic mice following removal of pups 8 days after the initiation of lactation. Luminal structures remain intact for at least 6 days after withdrawal of the pups, and there is a decrease in the number of apoptotic cells, suggesting a dramatic delay in tissue remodeling. Further studies are being carried out to further characterize this phenotype and to determine the role of Akt during involution.

Delayed Mammary Gland Involution in Transgenic Mice Expressing Activated Akt.

Steven M Anderson, Kathryn L Schwertfeger, Monica M Richert, Robert Strange. Pathology; Molecular Biology, University of Colorado Health Sciences Center; AMC Cancer Research Center, Denver, CO.

Apoptosis is known to occur in the mammary gland at several developmental time points including in the terminal end buds during puberty and, most dramatically, during involution following weaning. It is not clear what signaling molecules regulate apoptosis during involution. Although several studies have indicated that changes in the levels of Bcl2 family members might be important, a consistent pattern has not emerged. It is not clear whether increases in pro-apoptotic or decreases in anti-apoptotic Bcl2 family members are most important. We have focused upon the anti-apoptotic serine/threonine protein kinase Akt (also known as protein kinase B) which is activated by insulin-like growth factor I, epidermal growth factor, and prolactin all of which are important in regulating mammary gland development. Using immunoblot analysis, we show that Akt is expressed in the virgin, pregnant, lactating, and involuting mammary gland. To examine the role of Akt in regulating mammary gland involution we have generated transgenic mice which express a constitutively activated mutant of Akt referred to as Myr-Akt under the control of the mouse mammary tumor virus promoter. Founder mice were established and the expression of the transgene has been determined using both RT-PCR and immunoblot analysis. Initial studies suggest that involution is delayed in transgenic mice following forced weaning. Secretory alveolar structures remain intact for at least 6 days after withdrawal of the pups, and there is a delay in the onset of epithelial cell apoptosis, demonstrating an effect of activated Akt on tissue remodeling. A less dramatic phenotype was also observed in a second transgenic line corresponding to a reduced level of transgene expression. In addition to suppressed involution, there may be an effect on lactation since the growth of pups nursed by transgenic females (as determined by weight) as decreased by nearly 50% compared to litters nursed by normal females over the first eight days of life. These data suggest that Akt is a critical regulator of mammary involution and may also influence lactation.